Cytochrome *o* Type Oxidase from *Escherichia coli*. Characterization of the Enzyme and Mechanism of Electrochemical Proton Gradient Generation†

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ABSTRACT: Cytochrome *o* type oxidase purified from the membrane of *Escherichia coli* consists of four polypeptides (M_r 66 000, 35 000, 22 000, and 17 000), and the monomeric form predominates in octyl β-D-glucopyranoside. The oxidase complex contains two *b*-type cytochromes (*b*-558 and *b*-563) and 2 mol of heme/mol of enzyme. Cytochrome *o* utilizes ubiquinol-1 and a number of other artificial electron donors as substrates but does not oxidize reduced cytochrome *c* or ferrocyanide. Activity is highly dependent upon exogenous phospholipids and/or Tween 20, and the quinone analogues 2-heptyl-4-hydroxyquinoline *N*-oxide and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole are potent inhibitors. Proteoliposomes were formed by detergent dilution or dialysis in the presence of the oxidase and phospholipids, followed by freeze–thaw/sonication. Vesicles formed by this means are unilamellar and contain a random distribution of 85–90-Å intramembranous particles on the convex and concave fracture surfaces. During oxidase turnover, the reconstituted system generates a proton electrochemical gradient (interior negative and alkaline) of −115 to −140 mV; however, respiratory control is minimal (i.e., respiratory control ratios of about 1.5 are observed). By using a glass electrode to measure changes in external pH and the fluorescence of entrapped 8-hydroxy-1,3,6-pyrenetrisulfonate to measure changes in internal pH, it is apparent that during ubiquinol oxidation, protons are released on the external surface of the membrane and consumed on the internal surface. In contrast, with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, an electron donor that carries few protons at neutral pH, little change in external pH is observed until the protonophore carbonyl cyanide *m*-chlorophenylhydrazone is added, at which point the medium becomes alkaline. The results taken as a whole are consistent with the concept that oxidase turnover generates an electrical potential (interior negative) due to vectorial electron flow from the outer to the inner surface of the membrane. The pH gradient (interior alkaline), on the other hand, appears to result from scalar (i.e., nonvectorial) reactions that consume and release protons at the inner and/or outer surfaces of the membrane, respectively. In other words, cytochrome *o* oxidase from *Escherichia coli* does not appear to catalyze vectorial proton translocation.

In its most general form, the chemiosmotic concept of Mitchell (1961, 1963, 1966, 1968) postulates that the basic energy-yielding processes of living cells—respiration or absorption of light—lead to the generation of a transmembrane electrochemical gradient of protons that is the immediate driving force for a number of seemingly unrelated reactions which include active transport and oxidative phosphorylation or photophosphorylation [cf. Nichols (1982) and Kaback (1983)]. The proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] is composed of electrical and chemical parameters according to the following relationship:

$$\frac{\Delta\bar{\mu}_{H^+}}{F} = \Delta\psi - \frac{2.3RT}{F}\Delta\text{pH} \qquad (1)$$

where $\Delta\psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentrations across the membrane (R is the gas constant, T is the absolute temperature, and F is the Faraday constant; $2.3RT/F$ is equal to 58.8 at room temperature).

Over the past 10–15 years, a large body of evidence has accumulated demonstrating virtually unequivocally that the basic tenets of the chemiosmotic hypothesis are correct [cf. Skulachev & Hinkle (1981)]. Thus, many types of energy-coupling membrane systems generate a $\Delta\bar{\mu}_{H^+}$ of significant magnitude, and it has been shown that this thermodynamic entity is involved in a wide array of phenomena from bacterial motility to uptake and storage of neurogenic amines in the adrenal medulla [cf. Kaback (1984)].

In aerobically growing *Escherichia coli*, as in mitochondria, $\Delta\bar{\mu}_{H^+}$ is generated primarily by substrate oxidation via a membrane-bound respiratory chain with oxygen as the terminal electron acceptor. Although the molecular mechanism of proton translocation is unsolved, Mitchell (1966, 1968) has postulated that "loops" in the respiratory chain may be an important means of proton translocation across the membrane during electron flow. According to this notion, the electron and proton carriers that comprise the membrane-bound respiratory chain are disposed alternatively and asymmetrically across the membrane in such a manner that protons move from one side of the membrane to the other with a net flux of electrons in the opposite direction. In addition, Mitchell (1976) has postulated the existence of a "protonmotive ubiquinone cycle" in order to explain the absence of appropriate proton carriers after cytochrome *b* in the mitochondrial respiratory chain.

Given this type of scheme, it is apparent that the "protonmotive stoichiometry" (i.e., the stoichiometry between proton extrusion and electron transfer) in the respiratory chain cannot be greater than 2, since the known proton carriers do not react with more than two protons during a single turnover. Although Mitchell's laboratory and others [cf. Mitchell &

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\psi$, membrane potential; ΔpH, pH gradient; octyl glucoside, octyl β-D-glucopyranoside; PMS, phenazine methosulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium (bromide salt); pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; diS-C_3-(5), 3,3′-diisopropylthiodicarbocyanine; diBAc_2-(5), oxonol VI; SOD, superoxide dismutase; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; DCI, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Q_1H_2, ubiquinol-1; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; KP_i, potassium phosphate; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine.

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Moyle (1979)] have reported stoichiometries of 2, the findings have been challenged by Lehninger and co-workers and a number of other investigators who report values of 3 or 4 (Brand et al., 1976; Lawford, 1977; Papa et al., 1970; Reynafarje et al., 1976, 1978; Sigel & Carafoli, 1978; Wikström & Saari, 1977; Wikström & Krab, 1979). Furthermore, purified mitochondrial cytochrome oxidase [cf. Wikström & Krab (1979)]] and terminal oxidases purified from *Paracoccus denitrificans* (Solioz et al., 1982), the thermophile PS-3 (Sone & Hinkle, 1982), and *Thermus thermophilus* (Sone et al., 1983) appear to catalyze vectorial proton, as well as electron, translocation in the absence of proton carriers. On the other hand, terminal oxidases purified from *Rhodopseudomonas spheroides* (Gennis et al., 1982) and *Nitrobacter agilis* (Sone et al., 1983) do not seem to translocate protons vectorially when incorporated into proteoliposomes. In any event, the question of whether or not terminal oxidases catalyze vectorial proton translocation is highly controversial at present [cf. Mitchell & Moyle (1983), Casey & Azzi (1983), and Papa et al. (1983a)].

In previous communications (Kita et al., 1982; Matsushita et al., 1983), purification of the *o*-type cytochrome oxidase from *E. coli* was described, and it was demonstrated that turnover of this enzyme generates both a $\Delta\psi$ (interior negative) and a ΔpH (interior alkaline) when reconstituted into proteoliposomes. Furthermore, when proteoliposomes are prepared with the oxidase and purified *lac* carrier protein, they transport lactose against a concentration gradient in a fashion that mimics intact cells and right-side-out membrane vesicles (Matsushita et al., 1983). In this paper, we present a detailed characterization of cytochrome *o* oxidase and examine the mechanism of $\Delta\bar{\mu}_{H^+}$ generation. The results are consistent with the ideas expressed originally by Mitchell (1966, 1968). That is, although turnover of cytochrome *o* leads to the formation of both a $\Delta\psi$ and a ΔpH, the oxidase does not appear to translocate protons vectorially.

Materials and Methods

Materials

Phospholipids were prepared from *E. coli* B as described (Newman & Wilson, 1980). Octyl β-D-glucopyranoside (octylglucoside), valinomycin, nigericin, phenazine methosulfate (PMS), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Calbiochem. [^{3}H]Tetraphenylphosphonium (TPP^+) was synthesized by the Isotope Synthesis Group of Hoffmann-La Roche, Inc., under the direction of Dr. A. Liebman. [^{3}H]Acetate (sodium salt) and $^{14}CSCN^-$ (potassium salt) were obtained from New England Nuclear and Amersham/Searle, respectively. 8-Hydroxy-1,3,6-pyrenetrisulfonate (pyranine) was purchased from Eastman-Kodak, and 3,3′-diisopropylthiodicarbocyanine [diS-C_3-(5)] and oxonol VI [diBAc_2-(5)] were from Molecular Probes. Superoxide dismutase (SOD), *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), dichlorophenolindophenol (DCI), phenylmethanesulfonyl fluoride, and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were obtained from Sigma. Ubiquinone-1 was obtained from Hoffmann-La Roche, Inc., and ubiquinol-1 (Q_1H_2) was prepared by the method of Rieske (1967). 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was the generous gift of Dr. B. Trumpower.

Methods

Growth of Cells and Membrane Preparation. *E. coli* GR19N (*cyd*$^-$) (Green & Gennis, 1983) was grown aerobically into late logarithmic phase in minimal medium A (Davis & Mingioli, 1950) containing 0.5% sodium lactate and 0.15% casamino acids (pH 7.0). Cells were harvested and passed through a French pressure cell, and the membrane fraction was recovered as described (Matsushita et al., 1983).

Right-side-out membrane vesicles were prepared by osmotic lysis (Kaback, 1971; Short et al., 1975).

Purification of Cytochrome o. After GR19N membranes were sequentially extracted with 5 M urea and 6% cholate, cytochrome *o* was solubilized in 1.25% octyl glucoside in the presence of *E. coli* phospholipids and chromatographed on DEAE-Sepharose CL-6B as described (Matsushita et al., 1983). Where indicated, purified oxidase was then subjected to ammonium sulfate fractionation and gel filtration through Sephacryl S-200 as follows: To pooled DEAE-Sepharose fractions containing Q_1H_2 oxidase activity, solid ammonium sulfate was added to 60% saturation, and the mixture was stirred for 10 min on ice. After centrifugation at $10000g_{max}$ for 10 min, a small yellow pellet and an orange supernatant were observed. The supernatant was decanted, and solid ammonium sulfate was added to 90% saturation. After 10-min incubation on ice with stirring, followed by 10-min centrifugation at $10000g_{max}$, a red precipitate floating on the surface of the sample was obtained. The precipitate was collected and dissolved in 50 mM potassium phosphate (KP_i, pH 7.5) containing 1% octyl glucoside (1–2-mL final volume). The concentrated sample was applied to a Sephacryl S-200 column (1.5 × 83 cm) that had been preequilibrated with 50 mM KP_i (pH 7.5) containing 1% octyl glucoside. The column was eluted with the same buffer at a flow rate of about 5 mL/h, and Q_1H_2 oxdiase activity and protein chromatographed as a single coincident peaks.

Reconstitution of Proteoliposomes Containing Purified Cytochrome o. Proteoliposomes with a protein:lipid ratio of 1:125 (w/w) were prepared essentially as described (Matsushita et al., 1983). Purified oxidase (0.5 mg of protein in 50 mM KP_i, pH 7.5) was mixed with sonicated *E. coli* phospholipids (62.5 mg of lipid in 50 mM KP_i, pH 7.5), and octyl glucoside was added to a final concentration of 1.25%. The mixture was incubated on ice for 10–20 min and then diluted into 30 volumes of 50 mM KP_i (pH 7.5) that had been equilibrated to room temperature. After the mixture was stirred with a magnetic bar for 10 min, proteoliposomes were collected by centrifugation at $120000g_{max}$ for 2 h. The supernatant was discarded, and the proteoliposomes were resuspended in 50 mM KP_i (pH 7.5) to a protein concentration of 200–250 μg/mL and frozen rapidly in liquid nitrogen. Just prior to use, the samples were thawed at room temperature and sonicated for 10–20 s with a bath-type sonicator (Matsushita et al., 1983).

In order to obtain proteoliposomes with a higher protein to lipid ratio (1:10 w/w), detergent dialysis was employed. Purified oxidase (0.5 mg of protein in 50 mM KP_i, pH 7.5) was mixed with sonicated *E. coli* phospholipids (5 mg of lipid in 50 mM KP_i, pH 7.5), and octyl glucoside was added to a final concentration of 1%. After the mixture stood on ice for 10–20 min, it was dialyzed twice against 150 volumes of 50 mM KP_i (pH 7.5) at 4 °C for a total of 20 h. Proteoliposomes were harvested by centrifugation, resuspended to a protein concentration of 1.5–1.7 mg/mL, and frozen in liquid nitrogen as described above. Where indicated, 50 mM choline phosphate (pH 7.5) was used in place of KP_i and asolectin (soybean phospholipids) in place of *E. coli* phospholipids.

Electron Microscopy. Proteoliposomes with a cytochrome *o* to lipid ratio of 1:10 (w/w) were frozen ultrarapidly in the

absence of fixatives or cryoprotectants (Costello, 1980). Replicas were formed by fracturing at −160 °C, followed by unidirectional deposition of platinum.

Measurement of $\Delta\psi$ and ΔpH. $\Delta\psi$ (interior negative) was determined either by measuring the fluorescence quenching of diS-C_3-(5), using valinomycin-mediated potassium diffusion potentials ($K^+_{in} \rightarrow K^+_{out}$) for quantitation (Waggoner, 1979; Kita et al., 1982; Matsushita et al., 1983), or by measuring the steady-state distribution of [^{3}H]TPP$^+$ by flow dialysis (Ramos et al., 1979). Where indicated, fluorescence quenching and flow dialysis experiments were also performed with di-BAc_2-(5) and $^{14}CSCN^-$ in order to detect $\Delta\psi$ (interior positive). ΔpH (interior alkaline) was determined from the steady-state distribution of [^{3}H]acetate by using flow dialysis (Ramos et al., 1979). For calculation of $\Delta\psi$ and ΔpH from flow dialysis experiments, the internal volume of the proteoliposomes was estimated from the trapped volume of [1-^{14}C]lactose or $^{86}Rb^+$ (Garcia et al., 1983). The proton electrochemical gradient ($\Delta\bar{\mu}_H{}^+$) was calculated from eq 1.

Determination of External and Internal pH. External pH was measured directly and continuously with a Radiometer pH meter (pHm84) connected to a Radiometer pH electrode (GK 2401 B) and a Radiometer chart recorder (REC 61 Servograph). The measurements were performed in a closed electrode vessel that was jacketed and maintained at 25 °C during the experiments. The vessel was continuously flushed with a stream of water-saturated argon, and additions were made by means of a lateral inlet. Reaction mixtures (2.5 mL final volume) contained 150 mM KCl, 80 μM Q_1H_2 or 1 mM TMPD, 2 μM valinomycin (where indicated), and 5 μL of proteoliposomes at a final buffer concentration of 0.1 mM KP_i (pH 7.5). The pH was adjusted to pH 6.8–7.0 with KOH. Reactions were initiated by addition of 20 μL of air-saturated 150 mM KCl (9.96 nanoatom of O_2) (oxygen-pulse method). Calibration of the measured pH changes was performed at the conclusion of the experiments by adding aliquots of 1 mM HCl. In addition, where indicated, external pH measurements were made by using a reductant-pulse method. The conditions used for these determinations were identical with those described above except that the reaction mixture was flushed continuously with a stream of water-saturated oxygen, and the reaction was initiated by addition of a given electron donor.

Internal pH was measured by following the fluorescence of pyranine (Clement & Gould, 1981) entrapped within the proteoliposomes. The reactions were performed at 25 °C in a Perkin-Elmer MPF-4 spectrophotofluorometer using excitation and emission wavelengths of 460 and 520 nm, respectively. Reaction mixtures (2 mL total volume) contained 15 mM KP_i (pH 7.15), 5 mM dithiothreitol (DTT), and 10 μL of proteoliposomes with entrapped pyranine. Proteoliposomes containing entrapped pyranine were prepared as follows: An aliquot (80 μL) of proteoliposomes with a protein to lipid ratio of 1:125 (w/w) was diluted with 320 μL of 5 mM monobasic KP_i, and 20 μL of 50 mM pyranine was added. The suspension was sonicated for 5 s in a bath-type sonicator (Matsushita et al., 1983), diluted with 10 mL of 15 mM KP_i (pH 7.15), and centrifuged at $120000g_{max}$ for 45 min. The supernatant was decanted, and the pellet was resuspended and washed once in 15 mM KP_i (pH 7.15). The proteoliposomes were then resuspended to 80 μL in 15 mM KP_i (pH 7.15).

Other Analytical Procedures. Oxidation of given electron donors was measured with an oxygen electrode at 25 °C. Reaction mixtures (1 mL final volume) contained 50 mM KP_i (pH 7.5) and given concentrations of enzyme or proteoliposomes and electron donors. The oxygen concentration at 25 °C was assumed to be 258 μM. Where indicated, Q_1H_2 oxidase activity was also measured spectrophotometrically as described (Matsushita et al., 1983).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 10–14% polyacrylamide gels as described (Matsushita et al., 1983). Gels were stained with Coomassie brilliant blue R-250 or with silver (Merril et al., 1981).

Sucrose density gradient centrifugation was performed with linear gradients from 5 to 20% sucrose prepared in 50 mM KP_i (pH 7.5) containing 1% octyl glucoside. The sample, which contained purified cytochrome *o* oxidase with catalase, yeast alcohol dehydrogenase, and lactoperoxidase as standards, was carefully pipetted on top of the gradient, and the sample was centrifuged at 40 000 rpm for 16 h in a Beckman SW65 rotor at 4 °C. After centrifugation, the gradient was fractionated, and each enzyme activity was measured by standard procedures.

Absorption spectrophotometry was performed with a Beckman DU-8 spectrophotometer. Cytochrome *o* content was calculated from the absorbance difference between 416 and 430 nm in the CO difference spectrum. Protein was assayed as described (Newman et al., 1981).

Results

Characterization of Cytochrome o Type Oxidase. As reported previously (Matsushita et al., 1983), cytochrome *o* is purified relatively easily from the cytochrome *d* deficient mutant GR19N (Green & Gennis, 1983) by sequential extraction of the membrane fraction with urea and cholate, followed by extraction with octyl glucoside and chromatography on DEAE-Sepharose CL-6B (Figure 1A, lanes 1–4). Although it is not readily apparent from Coomassie blue staining, there are minor impurities in the preparation after DEAE-Sepharose chromatography (cf. Figure 1A, lane 4) which can be removed by ammonium sulfate fractionation and gel filtration on Sephacryl S-200 (cf. lane 5). However, gel filtration chromatography lowers the specific activity of the enzyme from 100–120 to 70–80 units/mg of protein, even when the assays are carried out in the presence of Tween 20 and *E. coli* phospholipid (cf. below). In any event, before or after ammonium sulfate fractionation and gel filtration, the purified oxidase contains 7.5 nmol of cytochrome *o*/mg of protein (using a millimolar extinction coefficient of 170, rather than 80; cf. Discussion) and 17 nmol of heme *b*/mg of protein (i.e., 2 mol/mol of enzyme).

On SDS–PAGE of purified cytochrome *o*, two polypeptides are visualized with Coomassie blue, one at M_r 55 000 and the other at M_r 34 000 (Figure 1A, lanes 4 and 5). The use of a more sensitive silver stain, however, demonstrates that two additional lower molecular weight polypeptide species (M_r's of 22 000 and 17 000) are present in the terminal oxidase complex (Figure 1B, lane 4), a finding that is consistent with a recent immunological characterization (Kranz & Gennis, 1983). Notably, both of the lower molecular weight bands are observed when the oxidase is purified in the presence of 0.5 mM phenylmethanesulfonyl fluoride, and both of these bands are retained after ammonium sulfate fractionation and chromatography on Sephacryl S-200. In addition, antibody raised against the purified oxidase complex reacts with polypeptides I and II (M_r 55 000 and 34 000, respectively) but not with polypeptides III and IV (M_r 22 000 and 17 000, respectively), as judged by immunoblotting experiments [data not shown; cf. Carrasco et al. (1982)]. It seems unlikely, therefore, that polypeptides III and IV result from proteolytic degradation of the larger polypeptides.

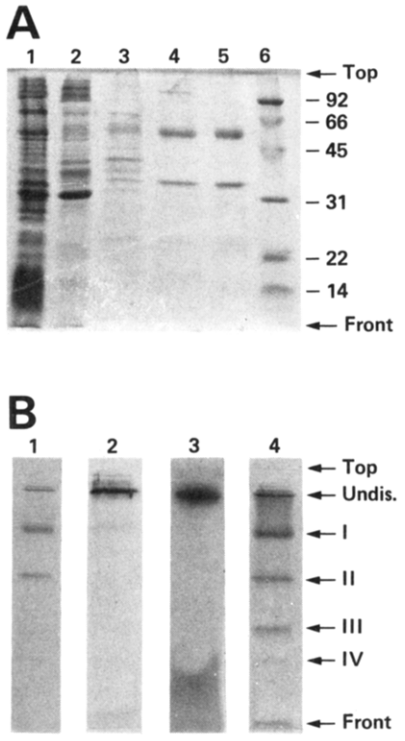

FIGURE 1: SDS–PAGE of cytochrome *o* complex. Samples were treated with 3% SDS and 100 mM DTT for 30 min at room temperature and electrophoresed in 12.5% polyacrylamide gels containing 0.1% SDS as described under Methods. (A) Coomassie blue stained gel: lane 1, crude membrane fraction (20 μg of protein); lane 2, membrane fraction after sequential extraction with 5 M urea and 6% cholate (12 μg of protein); lane 3, octyl glucoside extract from urea/cholate-treated membranes (3.9 μg of protein); lane 4, pooled DEAE-Sepharose fractions containing cytochrome *o* (4.4 μg of protein); lane 5, pooled Sephacryl S-200 fractions after dialysis against 50 mM NaP_i (pH 7.5) containing 0.1% SDS (5.7 μg of protein); lane 6, molecular weight markers (phosphorylase *b*, 92.5K; bovine serum albumin, 66.2K; ovalbumin, 45K; carbonic anhydrase, 31K; trypsin inhibitor, 21.5K; lysozyme, 14.4K; 1 μg of protein each). (B) Pooled Sephacryl S-200 fractions prior to dialysis: lane 1, Coomassie blue stain (4 μg of protein); lane 2, heme-catalyzed peroxidase activity (Thomas et al., 1976) (4 μg of protein); lane 3, TMPD oxidase activity (Kranz & Gennis, 1983) (4 μg of protein); lane 4, silver stain (2.3 μg of protein).

When purified oxidase is subjected to SDS–PAGE prior to removal of KP_i and octyl glucoside, an undissociated form of the enzyme is observed which migrates at M_r >100000 (Figure 1B). The mobility of this band is dependent on the concentration of polyacrylamide used during electrophoresis, and quantitation yields a molecular weight of about 140000 (Figure 2). Dialysis of the enzyme against 50 mM NaP_i (pH 7.5) containing 0.1% SDS leads to disappearance of the band (compare Figure 1A, lane 5, with Figure 1B, lane 1). Importantly, moreover, the band exhibits heme-catalyzed peroxidase activity (Thomas et al., 1976) (Figure 1B, lane 2) and TMPD oxidase activity (Kranz & Gennis, 1983) (Figure 1B, lane 3), while band I stains faintly for heme but does not oxidize TMPD.

As shown in Figure 2, the electrophoretic mobility of band I in SDS is highly dependent on the concentration of acrylamide used in the gel, while the mobilities of bands II, III, and IV are relatively unaffected by acrylamide concentration. From the retardation coefficients (Figure 2, inset), the molecular weights of bands I, II, III, and IV are estimated to be 66000, 35000, 22000, and 17000, respectively.

The molecular weight of cytochrome *o* under more "native" conditions was examined by using sucrose density gradient centrifugation in the presence of 1% octyl glucoside (Figure 3). As shown, two peaks are observed, both of which exhibit Q_1H_2 oxidase activity. The major peak sediments with a density that is essentially identical with that of alcohol dehydrogenase (M_r 140000) and is most probably the monomeric form of the oxidase. The second, smaller peak which sediments at a density slightly lower than that of catalase (M_r 240000) probably represents a dimeric form of the oxidase.

Table I: Substrate Specificity of Cytochrome *o* Oxidase[a]

substrate	concn (μM)	act. (microatom of O_2 min^{-1} mg^{-1})	K_m (μM)
Q_1H_2	80	84.0	10
TMPD	2500	5.8	4200
DAD	2500	13.5	
PMS	10	23.5	
DCI	100	2.9	

[a] Oxidase activity was measured in the presence of 0.1% Tween 20 and 0.25 mg/mL *E. coli* phospholipid by using an oxygen electrode as described under Methods. DTT (5 mM) or ascorbate (10 mM), respectively, was used to reduce Q_1H_2 or TMPD, DAD, PMS, and DCI.

Table II: Inhibitors of Cytochrome *o* Oxidase[a]

inhibitor	concn (μM)	inhibition (%)	K_i (μM)
KCN	200	89.6	23
HQNO	3.3	77.3	0.8
UHDBT	0.3	76.8	0.3

[a] Proteoliposomes with a cytochrome *o* to lipid ratio of 1:10 (w/w) were prepared, and Q_1H_2 oxidase activity was measured spectrophotometrically as described under Methods. Samples were incubated with a given inhibitor for 1–2 min at room temperature prior to addition of Q_1H_2. Although not shown, similar results were obtained with TMPD as substrate.

The activity of purified cytochrome *o* is highly dependent upon the presence of detergent and/or phospholipid, particularly after gel filtration (Figure 4). *E. coli* phospholipid stimulates Q_1H_2 oxidase activity about 20-fold at a concentration of 0.25 mg/mL, and although not shown, asolectin has the same effect. Tween 20 also stimulates partially when added in place of phospholipid, and maximum activity is observed in the presence of phospholipid and 0.1% Tween 20.

Purified cytochrome *o* catalyzes the oxidation of a variety of electron donors with redox potentials between +50 and +260 mV (Table I). However, it exhibits no activity whatsoever toward ascorbate, ferrocyanide, or cytochrome *c* from horse or yeast. Relative to cytochrome *d* (Miller & Gennis, 1983), the *o*-type oxidase has a much lower K_m for Q_1H_2 (10 μM vs. 100 μM) but a higher K_m for TMPD (4.2 mM vs. 0.68 mM). In addition, cytochrome *o* is markedly inhibited by cyanide and also by the ubiquinone analogues HQNO and UHDBT with either Q_1H_2 (Table II) or TMPD (not shown) as electron donors. Kinetically, the quinol analogues exhibit a mixed type of inhibition with respect to Q_1H_2 concentration (data not shown).

Cytochrome *o* contains two *b*-type cytochromes, as evidenced by the α bands at 558 and 563 nm in the reduced spectrum (Figure 5A). Furthermore, exposure of the reduced oxidase to CO causes the γ band to split into two peaks at 419 and 426 nm and yields a CO difference spectrum that is typical of cytochrome *o* (Figure 5B) (Wallace & Young, 1977; Yang & Jurtshuk, 1978).

Electron Microscopy of Proteoliposomes Reconstituted with Cytochrome o. As shown previously with purified *lac* carrier protein (Garcia et al., 1983; Kaback, 1983), proteoliposomes formed by octyl glucoside dilution followed by freeze–thaw/sonication are unilamellar vesicles that exhibit no internal structure. Relatively low magnification electron microscopy of platinum replicas of freeze-fractured proteolipo-

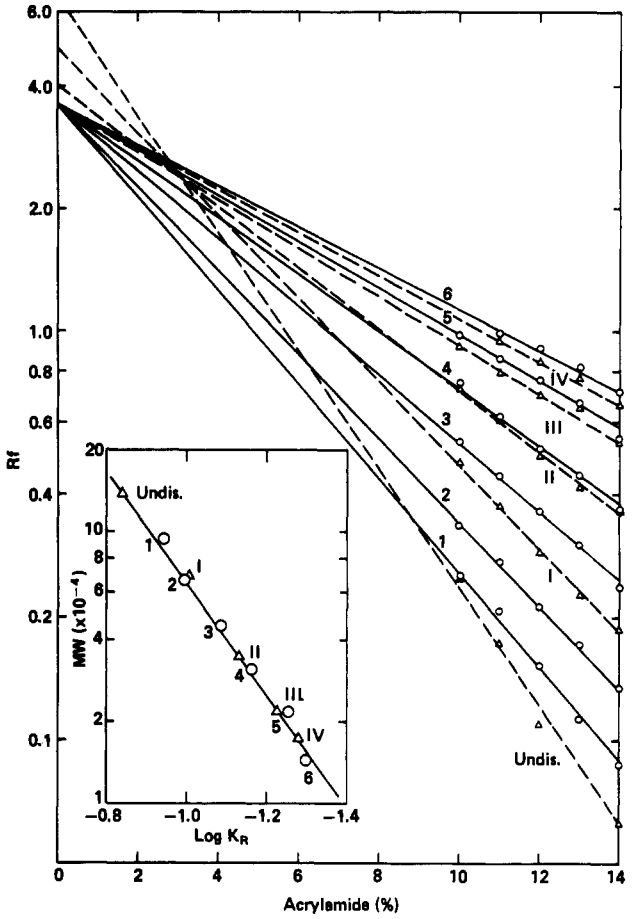

FIGURE 2: Effect of acrylamide concentration on electrophoretic mobility of cytochrome *o* complex and its component polypeptides. SDS–PAGE was performed separately in 10, 11, 12, 13, and 14% polyacrylamide gels which were then stained with silver as described under Methods. The relative mobility (R_f) of each band and those of molecular weight standards were plotted against the percent acrylamide used. The solid lines (numbered 1, 2, 3, 4, 5, and 6) represent phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme, respectively. The broken lines (labeled Undis. and I, II, III, and IV) represent undissociated cytochrome *o* and polypeptides I, II, III, and IV, respectively. Inset: The retardation coefficient (K_R) of each polypeptide was calculated as described by Neville (1971) and plotted against the log of the molecular weight ($M_r \times 10^{-4}$). (O) Molecular weight standards; (Δ) undissociated cytochrome *o* and polypeptides I, II, III, and IV.

somes containing purified cytochrome *o* demonstrates that the great majority of these vesicles are also unilamellar (Figure 6a). Higher magnification reveals that both the convex and concave fracture surfaces exhibit a relatively uniform distribution of particles that are 85–90 Å in diameter (Figure 6b). Furthermore, all of the particles visualized are observed within fracture faces, indicating that most, if not all, of the oxidase is associated with the proteoliposomes.

Generation of $\Delta\psi$. As documented previously (Kita et al., 1982; Matsushita et al., 1983), proteoliposomes reconstituted with cytochrome *o* generate a $\Delta\psi$ (interior negative) during turnover of the oxidase. The conclusion is confirmed and extended by the data presented in Figure 7 which demonstrate that addition of Q_1H_2 to proteoliposomes (panel A) or right-side-out membrane vesicles (panel B) containing cytochrome *o* quenches the fluorescence of the carbocyanine dye diS-C_3-(5). Furthermore, quenching is enhanced considerably, particularly with proteoliposomes, when nigericin is added. Nigericin is an ionophore that catalyzes electrically neutral exchange of protons for K^+ (or Na^+), thereby collapsing ΔpH with a compensatory increase in $\Delta\psi$ (Ramos et al., 1976; Ramos & Kaback, 1977; Reenstra et al., 1980). On the other hand, immediately after addition of valinomycin, the signal returns rapidly to the base line, an observation that is consistent

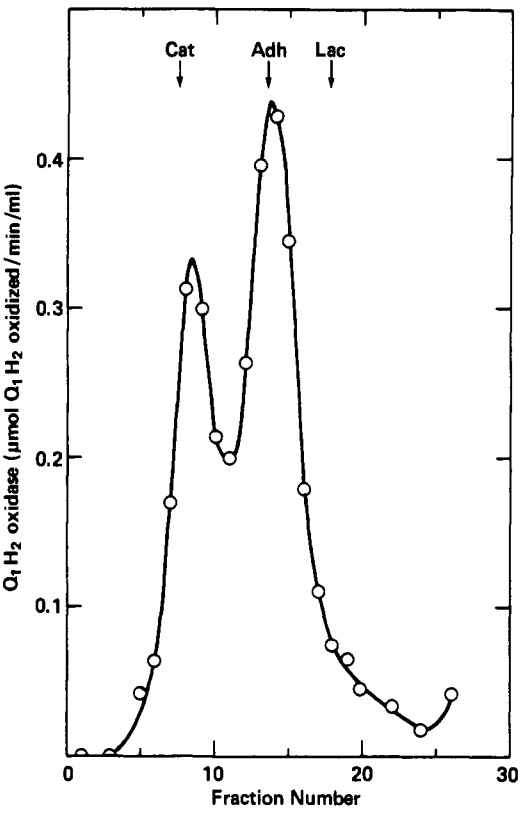

FIGURE 3: Sucrose density gradient centrifugation of purified cytochrome *o* oxidase. Cytochrome *o* oxidase after DEAE-Sepharose chromatography and ammonium sulfate fractionation was subjected to sucrose density gradient centrifugation as described under Methods. The arrows designate the positions of catalase (Cat, M_r 240K), yeast alcohol dehydrogenase (Adh, M_r 140K), and lactoperoxidase (Lac, M_r 78K).

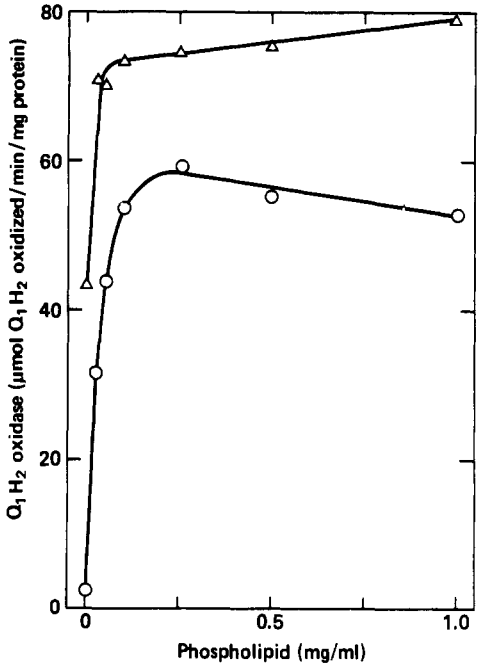

FIGURE 4: Effect of Tween 20 and/or phospholipids on cytochrome *o* oxidase activity. Cytochrome *o* oxidase purified through Sephacryl S-200 gel filtration chromatography (0.74 μg of protein/sample) was mixed with *E. coli* phospholipids in the absence (O) and presence (Δ) of 0.1% Tween 20 prior to addition of Q_1H_2. Enzyme activity was followed spectrophotometrically as described under Methods.

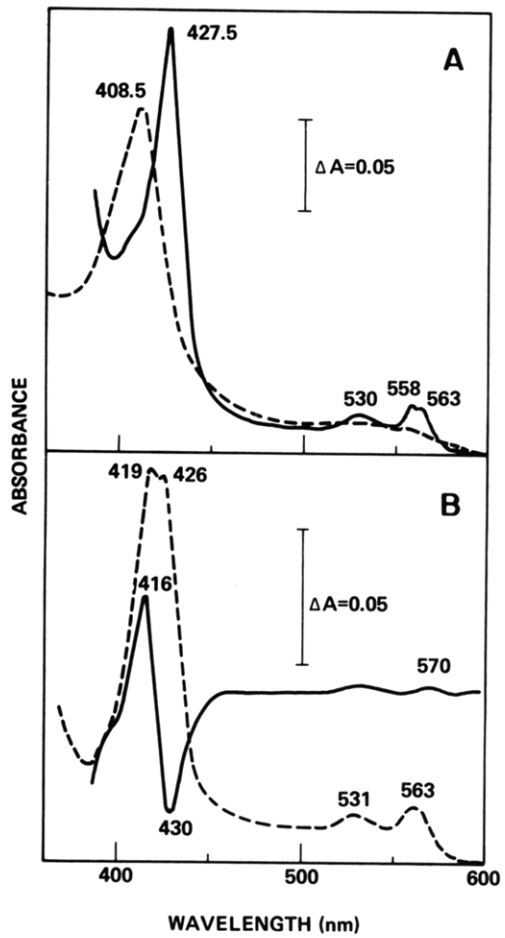

FIGURE 5: Absorption spectra of purified cytochrome *o* oxidase. (A) Absolute spectra of the oxidized (---) and dithionite-reduced (—) forms of the enzyme. Spectra were recorded at a protein concentration of 73 μg/mL as described under Methods. (B) CO spectra. After the sample (60 μg of cytochrome *o*/mL) was reduced with dithionite, CO was bubbled through the solution for 1 min, and after 30 min, the spectra were recorded. (---) Absolute spectrum of enzyme treated with dithionite and CO; (—) reduced + CO *minus* reduced difference spectrum.

with the ability of this ionophore to collapse $\Delta\psi$ in the presence of K^+ (Ramos et al., 1976; Ramos & Kaback, 1977; Reenstra et al., 1980). The findings provide a strong indication that during turnover, cytochrome *o* oxidase generates a significant ΔpH (interior alkaline) in addition to $\Delta\psi$.

Q_1H_2-induced quenching of diS-C_3-(5) is a linear function of the concentration of proteoliposomes in the cuvette up to about 1.2 μg of protein (Figure 8A). Furthermore, the percent quenching increases linearly up to about 3–4 μM Q_1H_2 and remains constant at Q_1H_2 concentrations exceeding about 8 μM (Figure 8B). The magnitude of the $\Delta\psi$ generated during oxidation of Q_1H_2 can be estimated by comparison to standard curves constructed from experiments in which fluorescence quenching is induced by imposition of K^+ diffusion gradients ($K^+_{in} \rightarrow K^+_{out}$) of known magnitude in the presence of valinomycin (Figure 8C). With proteoliposomes containing cytochrome *o* and *E. coli* phospholipids at a ratio of 1:125 (w/w), $\Delta\psi$ values of about −80 and −115 mV are calculated before and after the addition of nigericin, respectively (cf. Figure 7A).

Generation of $\Delta\psi$ (interior negative) can also be demonstrated with flow dialysis by measuring the distribution of [^{3}H]TPP^+ (Ramos et al., 1979) (Figure 9). As shown, addition of Q_1H_2 (panel A) or TMPD (panel B) results in a decrease in the dialyzable concentration of the lipophilic cation, and the effect is enhanced upon addition of nigericin. Addition of valinomycin, in contrast, causes release of [^{3}H]TPP^+, and the level of radioactivity in the dialysate returns to that of the control.

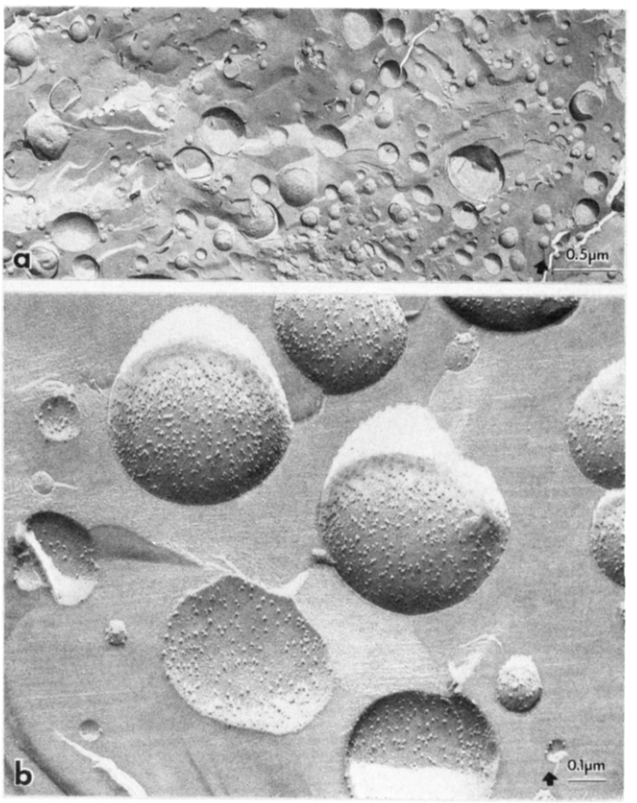

FIGURE 6: Freeze–fracture images of proteoliposomes containing cytochrome *o* oxidase. Proteoliposomes with an oxidase to phospholipid ratio of 1:10 (w/w) were frozen ultrarapidly in the absence of fixatives or cryoprotectants as described (Costello, 1980). Replicas were formed by fracturing at −160 °C, followed by unidirectional deposition (arrows) of platinum. (a) Survey of the preparation illustrating that the majority of the vesicles are single-walled. (b) High magnification view showing random distribution of 85–90-Å intramembranous particles on convex and concave fracture surfaces.

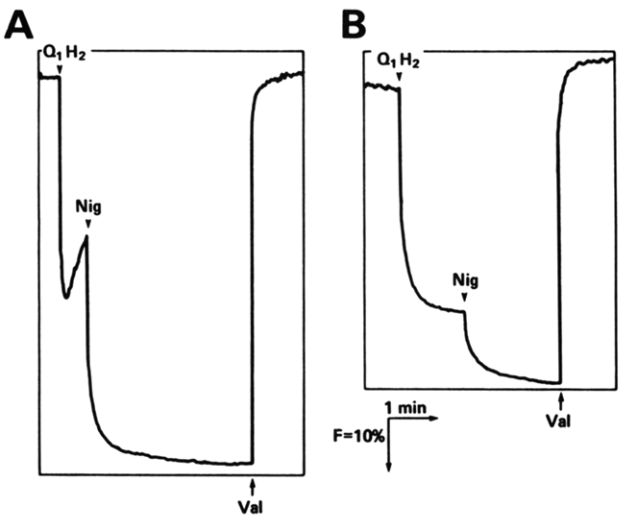

FIGURE 7: Fluorescence quenching of diS-C_3-(5) in proteoliposomes containing cytochrome *o* oxidase (A) and right-side-out membrane vesicles from *E. coli* GR19N (B). Reaction mixtures contained 50 mM KP_i (pH 7.5), 5 mM DTT, 1 μM diS-C_3-(5), and proteoliposomes (1.16 μg of protein) or right-side-out membrane vesicles (6.0 μg of protein) as indicated. Reactions were initiated by addition of 16 μM Q_1H_2, followed by addition of 0.025 μM nigericin (Nig) and 1 μM valinomycin (Val). (A) Proteoliposomes with a cytochrome *o* to phospholipid ratio of 1:125 (w/w); (B) right-side-out membrane vesicles.

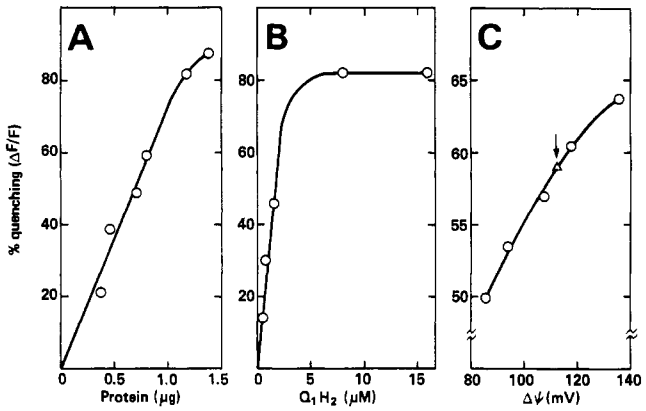

FIGURE 8: Q_1H_2-induced diS-C_3-(5) quenching in proteoliposomes containing cytochrome *o* oxidase. Proteoliposomes with a cytochrome *o* to phospholipid ratio of 1:125 (w/w) were prepared as described under Methods. (A) Relationship between diS-C_3-(5) quenching and concentration of proteoliposomes. Reaction mixtures contained 50 mM KP_i (pH 7.5), 5 mM DTT, 1 μM diS-C_3-(5), and proteoliposomes containing given amounts of cytochrome *o*. The experiments were performed as described in Figure 7. (B) Relationship between fluorescence quenching and Q_1H_2 concentration. Proteoliposomes (1.16 μg of protein) were used in the same reaction mixture as described in panel A, and the reactions were initiated by addition of given concentrations of Q_1H_2. (C) Calibration curve for estimation of Δψ. Fluorescence quenching of diS-C_3-(5) induced by valinomycin-mediated potassium diffusion potentials of various magnitudes was measured in proteoliposomes (0.81 μg of protein) as described under Methods (○). Q_1H_2-dependent quenching with the same amount of proteoliposomes (0.81 μg of protein) in the presence of nigericin corresponds to a Δψ of −115 mV (△).

Table III: Quantitation of Δψ (Interior Negative) and ΔpH (Interior Alkaline) in Proteoliposomes Containing Purified Cytochrome *o* Oxidase As Determined by Flow Dialysis[a]

substrate[b]	SOD[c]	Δψ (mV)		ΔpH (mV)	
		−Nig	+Nig	−Val	+Val
DTT/Q_1H_2	−	−62	−87	nd[d]	nd
	+	−86	−106	−36	−53
TMPD	+	−66	−94	−30	−46

[a] Proteoliposomes with a cytochrome *o* to phospholipid ratio of 1:125 (w/w) were prepared, and flow dialysis was performed as described under Methods, using a 0.2-mL reaction mixture containing 58 μg of protein/mL. Δψ was determined by measuring the steady-state distribution of [^{3}H]TPP$^+$ as described in Figure 9. ΔpH was determined by measuring the steady-state distribution of [^{3}H]acetate as described under Methods, using 37.5 μM [^{3}H]acetate (685 mCi/mmol). Where indicated, nigericin (Nig) or valinomycin (Val) was added at a final concentration of 0.25 or 2.5 μM, respectively. [b] DTT (5 mM) and Q_1H_2 (16 μM) or TMPD (2.5 mM) added to the reaction mixtures as indicated. [c] Superoxide dismutase (SOD; 300 units) was added to the reaction mixtures as indicated. [d] nd, not determined.

By measurement of the trapped volume of either ^{86}Rb or [1-^{14}C]lactose, the internal volume of the proteoliposomes can be determined (Garcia et al., 1983) and used to quantitate the Δψ generated (Table III). Relative to the values obtained from diS-C_3-(5) fluorescence quenching in the presence of Q_1H_2 (i.e., −80 and −115 mV, respectively, before and after addition of nigericin), the Δψ values obtained from flow dialysis appear to be significantly lower (i.e., −62 and −87 mV, respectively). However, since flow dialysis requires relatively high concentrations of proteoliposomes, and at high concentrations of the oxidase, noxious concentrations of superoxide are generated as a result of Q_1H_2 oxidation (Matsushita et al., 1983), flow dialysis was performed in the presence of superoxide dismutase (SOD) (Table III). Clearly, when the deleterious effects of superoxide anion are blocked by addition of the dismutase, the Δψ values obtained by flow dialysis are

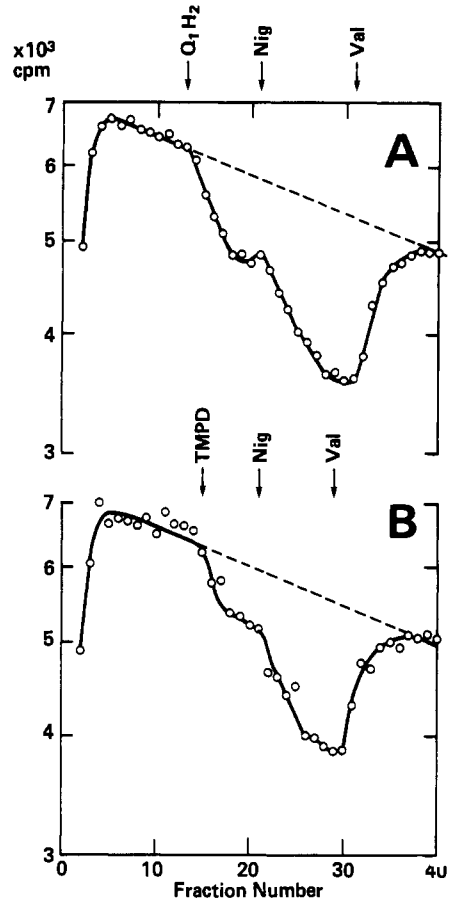

FIGURE 9: Flow dialysis determinations of [^{3}H]TPP$^+$ uptake by proteoliposomes reconstituted with cytochrome *o* oxidase. Flow dialysis was performed as described under Methods, using 0.2-mL reaction mixtures containing 50 mM KP_i (pH 7.5), 300 units of SOD, and 58 μg/mL protein [proteoliposomes with a cytochrome *o* to phospholipid ratio of 1:125 (w/w)]. Reactions were initiated by adding 20 μM [^{3}H]TPP$^+$ (2.5 Ci/mmol), and 5 mM DTT/16 μM Q_1H_2 (A) or 2.5 mM TMPD (B), 0.25 μM nigericin (Nig), and 2.5 μM valinomycin (Val) were added sequentially as indicated. (A) DTT/Q_1H_2; (B) TMPD.

essentially identical with those obtained with the fluorescence quenching technique. It is also noteworthy that TMPD oxidation in the presence of SOD leads to the generation of Δψ values of −66 and −94 mV, respectively, before and after addition of nigericin.

Although data are not presented, a few additional points should be mentioned regarding generation of Δψ in these preparations: (i) Although freeze–thaw/sonication is not absolutely necessary to prepare functional proteoliposomes, higher Δψ values are obtained after freeze–thaw/sonication. Thus, proteoliposomes prepared without freeze–thaw/sonication generate a Δψ of about −105 mV in the presence of Q_1H_2 and nigericin, while the same preparation generates Δψ values of about −115 and −140 mV, respectively, under the same conditions after one and three cycles of freeze–thaw/sonication. (ii) Data similar to those shown in Figure 7A are obtained with proteoliposomes prepared with choline in place of potassium. (iii) Proteoliposomes containing cytochrome *o* and asolectin, rather than *E. coli* phospholipids, exhibit properties similar to those presented in Figure 7A and 8, except that the magnitude of the Δψ generated is smaller, and enhancement of fluorescent quenching by nigericin is reduced. Possibly, the differences reflect enhanced permeability to protons in proteoliposomes prepared with asolectin, as opposed to *E. coli* phospholipids. (iv) When the anionic oxonol dye diBAc_2-(5) is used in place of diS-C_3-(5) or ^{14}CSCN$^-$ is used rather than

[^{3}H]TPP$^+$, no changes whatsoever are observed on addition of Q_1H_2.

ΔpH Generation. As discussed above, the observations that nigericin enhances the fluorescence quenching of diS-C_3-(5) and stimulates the uptake of [^{3}H]TPP$^+$ provide a strong indication that proteoliposomes reconstituted with cytochrome *o* generate a significant ΔpH (interior alkaline) in addition to $\Delta\psi$ during oxidation of Q_1H_2 or TMPD. Direct evidence supporting this contention is provided by flow dialysis experiments with [^{3}H]acetate, a permeant weak acid that accumulates in response to ΔpH (interior alkaline) (Ramos et al., 1976, 1979; Ramos & Kaback, 1977). As shown in Table III, proteoliposomes containing cytochrome *o* generate ΔpHs of about −36 and −30 mV, respectively, in the presence of Q_1H_2 and TMPD. Addition of valinomycin, which collapses $\Delta\psi$ with a compensatory increase in ΔpH (Ramos et al., 1976; Ramos & Kaback, 1977; Reenstra et al., 1980), causes significant increases in ΔpH with either electron donor.

By using a pH-sensitive glass electrode, changes in the external pH of lightly buffered suspensions of proteoliposomes containing cytochrome *o* were measured under various conditions (Figure 10). As shown in panel A, addition of Q_1H_2 to proteoliposomes under aerobic conditions (reductant-pulse method) causes acidification of the medium at a relatively slow rate (curve a). In the presence of valinomycin, however, the rate of acidification increases markedly, and the pH tracing reaches maximum displacement in about 1 min and then decreases slowly as the concentration of Q_1H_2 becomes limiting (curve b). Similarly, with the oxygen-pulse method (panel B), transient acidification is observed on injection of air-saturated KCl in the presence of valinomycin (curve a), and the phenomenon is completely abolished in the presence of CCCP (curve b).

TMPD is an electron carrier that is largely unprotonated at neutral pH (Papa et al., 1983b), and external pH measurements carried out with this electron donor are radically different from those observed with Q_1H_2 (Figure 10C). Addition of air-saturated KCl in the presence of TMPD and valinomycin under anaerobic conditions causes slight alkalinization of the medium at a slow rate (curve a). Dramatically, CCCP markedly accentuates this effect, and the medium becomes much more alkaline over the same time period (curve b).

Comparison of rates of acidification or alkalinization with rates of oxygen uptake in the presence of Q_1H_2 or TMPD permits calculation of H^+:O ratios under the conditions described. With Q_1H_2 as reductant, H^+:O ratios of greater than 1.5 have never been observed (values ranging from 0.9 to 1.4 were obtained in 10–15 independent experiments). With TMPD, net alkalinization in the presence of CCCP relative to oxygen consumption yields H^+:O ratios ranging from 1.4 to 1.7 (five to six independent experiments). Clearly, in both instances, the H^+:O ratios do not exceed 2, the theoretical value expected for a two-electron transfer from Q_1H_2 or TMPD to one oxygen atom.

As a corollary to the external pH measurements, changes in internal pH were monitored in proteoliposomes containing entrapped pyranine, an impermeant, pH-sensitive fluorophore (Clement & Gould, 1981) (Figure 11). Addition of Q_1H_2 causes an increase in fluorescence, indicating that the internal space becomes alkaline during turnover of the oxidase. Valinomycin causes a small increase in fluorescence, while nigericin induces immediate quenching, and the tracing returns rapidly to the base line. From a calibration curve constructed by titrating proteoliposomes with KOH in the presence of

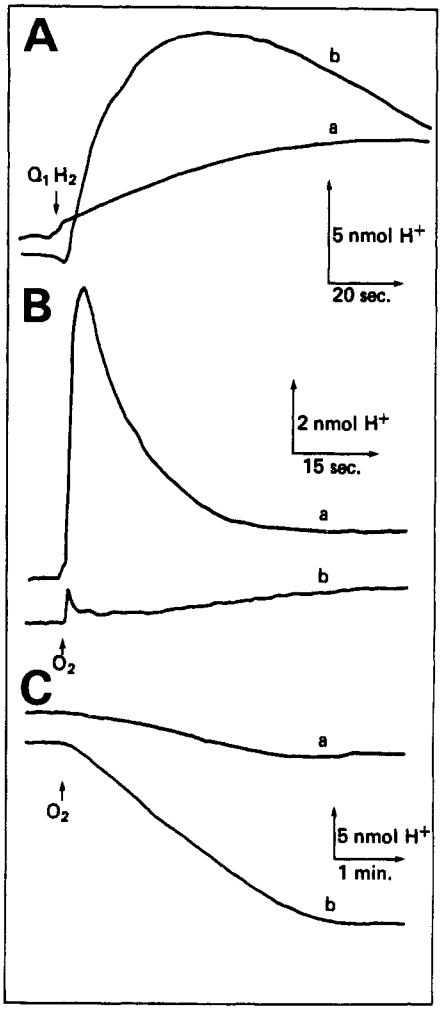

FIGURE 10: External pH changes during Q_1H_2 or TMPD oxidation in proteoliposomes containing cytochrome *o* oxidase. Details of the experimental conditions employed are described under Methods. (A) Reductant-pulse experiment: Reaction mixtures contained 150 mM KCl and proteoliposomes (2.23 μg of protein) with an oxidase to phospholipid ratio of 1:125 (w/w) (curve a), and where indicated (curve b), valinomycin was added to a final concentration of 0.8 μM. Reactions were initiated by addition of 36 μM Q_1H_2. (B) Oxidant-pulse experiment: Reaction mixtures contained 150 mM KCl, 80 μM Q_1H_2, 2 μM valinomycine, and proteoliposomes (7.85 μg of protein) with an oxidase to phospholipid ratio of 1:10 (w/w) without (curve a) and with (curve b) 2 μM CCCP. Reactions were initiated by addition of 9.96 nanoatom of oxygen. (C) Oxidant-pulse experiment: Reaction mixtures contained 150 mM KCl, 1 mM TMPD, 2 μM valinomycin, and proteoliposomes (7.0 μg of protein) with an oxidase to phospholipid ratio of 1:20 (w/w) without (curve a) and with (curve b) 2 μM CCCP. Reactions were initiated by addition of 9.96 nanoatom of oxygen.

nigericin, it is estimated that the fluorescence increase observed corresponds to an increase in the internal pH of 0.16–0.18 pH unit. Although this value is significantly less than that observed with flow dialysis, precise determinations of internal pH with pyranine are problematic (Clement & Gould, 1981). In any event, these observations in conjunction with the external pH measurements indicate that oxidation of Q_1H_2 causes the appearance of protons in the external medium, as well as their disappearance from the internal milieu. Changes in internal pH during TMPD oxidation cannot be measured with entrapped pyranine because the spectral properties of the electron donor interfere with the fluorescence measurements. However, since TMPD oxidation generates a ΔpH (interior alkaline) without acidifying the external medium, it is apparent that during TMPD oxidation protons are consumed on the inner surface of the membrane.

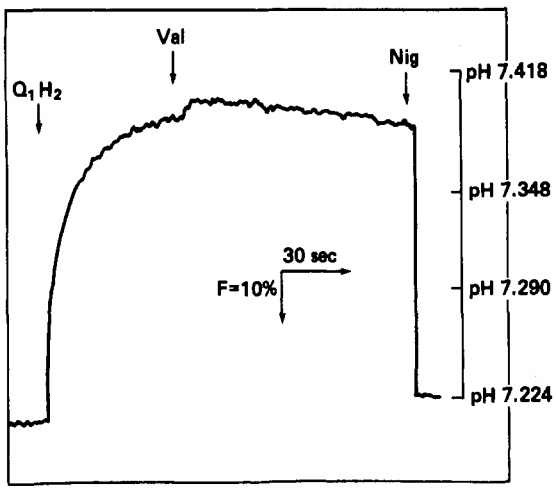

FIGURE 11: Internal pH changes induced by Q_1H_2 oxidation in proteoliposomes containing cytochrome *o* oxidase. Changes in internal pH were measured by following the fluorescence of pyranine (excitation, 460 nm; emission, 520 nm) entrapped in proteoliposomes as described under Methods. The reaction was initiated by addition of 80 μM Q_1H_2, and valinomycin (Val) and nigericin (Nig) were added sequentially to final concentrations of 0.5 and 0.063 μM, respectively, as indicated. Fluroescence changes were calibrated by titration with 1 N KOH in the presence of nigericin.

Respiratory Control. Despite the clear demonstration that proteoliposomes reconstituted with purified cytochrome *o* oxidase generate a relatively high $\Delta\bar{\mu}_{H^+}$, the preparations exhibit only a marginal degree of respiratory control. That is, the rate of Q_1H_2 oxidation is stimulated by only about 55% in the presence of valinomycin and nigericin (Figure 12). In the experiments shown, Q_1H_2 oxidase activity was measured at given temperatures in the absence and presence of the ionophores. Although the respiratory control ratio (i.e., Q_1H_2 oxidation rate in the presence of ionophores to Q_1H_2 oxidation rate in the absence of ionophores) exhibits slightly higher values at lower temperatures (i.e., maximum values of 1.50–1.55 are observed at 10–20 °C), variations in temperature do not cause a pronounced effect. In addition, it is noteworthy that the oxidase activity of the proteoliposomes exhibits a transition temperature at 20–22 °C which corresponds to the phase transition of the *E. coli* phospholipids used to prepare the proteoliposomes.[2]

Discussion

Characterization of Cytochrome o Type Oxidase. Cytochrome *o* oxidase purified from *E. coli* GR19N exhibits four polypeptide species on SDS–PAGE followed by silver staining, and the apparent molecular weights of the polypeptides are 66 000, 35 000, 22 000, and 17 000 after correction for the effect of acrylamide concentration on electrophoretic mobility. The sum of the molecular weights of the four polypeptides, 140 000, is similar to the molecular weight of the oxidase complex, as determined by sucrose density gradient centrifugation in the presence of octyl glucoside. On the other hand, a value of 130 000–140 000 is significantly higher than the minimum molecular weight of the complex, calculated from the heme content (two *b*-type hemes per mole), which is 118 000. Thus, with the exception of polypeptide I (M_r 66 000), which contains heme (Figure 1B), and polypeptide II (M_r 35 000), which is hydrolyzed by chymotrypsin with loss of oxidase activity (unpublished results), it cannot be concluded

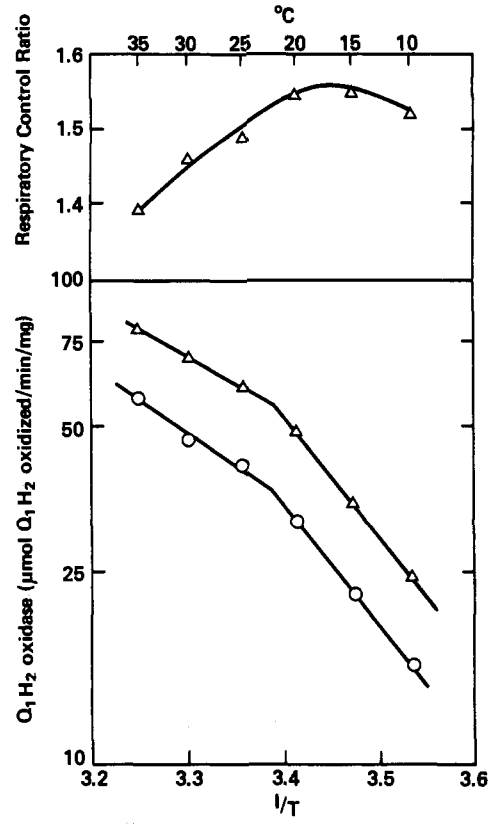

FIGURE 12: Effect of temperature on Q_1H_2 oxidase activity and on respiratory control in proteoliposomes containing cytochrome *o* oxidase. (Lower panel) Q_1H_2 oxidase activity was measured spectrophotometrically at given temperatures in proteoliposomes with an oxidase to phospholipid ratio of 1:125 (w/w) as described under Methods: (○) oxidase activity in the absence of ionophores: (△) oxidase activity in the presence of 2 μM valinomycin and 0.05 μM nigericin. (Upper panel) Respiratory control ratios were calculated from the activity observed in the presence of ionophores divided by the activity observed in the absence of valinomycin and nigericin. Experimental points are derived from data presented in the lower panel.

definitively that the other polypeptides are subunits of the oxidase, as opposed to contaminants that copurify with the enzyme. It is noteworthy, however, that each of the polypeptide species remains after further purification of the oxidase by ammonium sulfate fractionation and gel filtration chromatography and that the composition of the oxidase is unaffected by purification in the presence of a protease inhibitor. Furthermore, on the basis of immunoelectrophoretic studies (Kranz & Gennis, 1983), it has been concluded that the oxidase complex contains four subunits. Thus, until it is determined whether or not all four polypeptides are required for oxidase function, the tentative conclusion that the enzyme complex consists of four heterogeneous subunits seems justified.

Although it is apparent that the oxidase complex contains two *b*-type hemes, it is not clear that both hemes react with CO. From the CO difference spectrum, the CO-binding component of the oxidase complex is either 7.5 or 16 nmol/mg of protein, depending on whether a millimolar extinction coefficient of 170 (Daniel, 1970) or 80 (Wallace & Young, 1977), respectively, is used in the calculation. Obviously, the two values correspond almost exactly to one or two CO-binding components, respectively, in the oxidase complex. Importantly, in the absolute CO spectrum, split γ peaks are observed which may indicate that only one of the *b*-type hemes binds CO. If this is the case, a value of 170 would be the proper nanomolar extinction coefficient. This conclusion is not necessarily incompatible with recent potentiometric data indicating that both of the *b*-type hemes in cytochrome *o* have the same redox

[2] Liposomes prepared from *E. coli* lipids as described (Newman & Wilson, 1980) exhibit a phase transition at 20–22 °C, as determined by microcalorimetric analysis.

potential (Lorence et al., 1984), since the two heme components may be coupled thermodynamically.

Data presented earlier (Kita et al., 1982; Matsushita et al., 1983) and extended in this paper demonstrate that turnover of cytochrome *o* generates a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) of over −100 mV when the oxidase is incorporated into proteoliposomes. Moreover, the reconstituted system does not accumulate $diBAc_2$-(5) or $^{14}CSCN^-$, permeant lipophilic anions that respond electrophoretically to $\Delta\psi$ (interior positive). Thus, it seems reasonable to conclude that cytochrome *o*, like the *lac* carrier protein (Kaback, 1983), reconstitutes with a high degree of fidelity during octyl glucoside dilution. That is, a relatively high percentage of the oxidase molecules in the proteoliposomes probably have the same orientation as the oxidase in the native bacterial membrane. It should be emphasized, however, that the orientation of the oxidase molecules in the reconstituted membrane is unlikely to be quantitatively "right-side-out", particularly since the H^+:O ratio observed for Q_1H_2 falls significantly short of the theoretical value of 2. Clearly, a relatively small number of "inside-out" molecules could account for the discrepancy.

Mechanism of $\Delta\bar{\mu}_{H^+}$ Generation in Proteoliposomes Reconstituted with Cytochrome o Type Oxidase. As postulated in Mitchell's original concept of the mitochondrial aa_3-type terminal oxidase (Mitchell, 1968), the complex does not catalyze vectorial translocation of protons. Rather, the oxidase constitutes a "half-loop" in the mitochondrial membrane, whereby turnover generates $\Delta\psi$ (interior negative) as a result of vectorial electron flow from the outer to the inner surface of the membrane, and ΔpH (interior alkaline) is established because protons are consumed on the inner surface of the membrane during reduction of molecular oxygen to form water (Figure 13A). In other words, although the oxidase generates both components of $\Delta\bar{\mu}_{H^+}$, only the electrical component is generated via a vectorial reaction mechanism, while the pH gradient results from consumption of protons on the inner surface of the membrane. In contrast, recent experiments with purified terminal oxidase complexes from mitochondria (Wikström, 1981) *Paracoccus denitrificans* (Solioz et al., 1982), the thermophile PS-3 (Sone & Hinkle, 1982), and *Thermus thermophilus* (Sone et al., 1983) have challenged this notion by providing evidence that these oxidases may catalyze vectorial proton translocation in addition to vectorial electron flow (Figure 13B). The work presented here will be discussed in the context of these opposing viewpoints.

It is evident from previous studies (Kita et al., 1982; Matsushita et al., 1983) and from the present investigation that proteoliposomes reconstituted with cytochrome *o* generate a $\Delta\psi$ (interior negative) during oxidation of Q_1H_2. This property of the system is consistent with the idea that vectorial electron flow from the outer surface of the membrane to the inner surface can account for generation of $\Delta\psi$ but does not provide compelling support for this mechanism, since proton extrusion with or without vectorial electron flow in the opposite direction would also generate $\Delta\psi$. On the other hand, it is apparent from the studies presented in Figures 10 and 11 that during oxidase turnover in the presence of Q_1H_2, protons appear in the external medium and disappear from the internal space (Figure 13C). In themselves, these observations are also of little help because oxidation of Q_1H_2 on the outer surface of the membrane and consumption of protons on the inner surface to form water are scalar reactions either of which can lead to a ΔpH of appropriate polarity. Importantly though, if the oxidase acts as a proton pump in addition to catalyzing the scalar release of protons from Q_1H_2, the stoichiometry

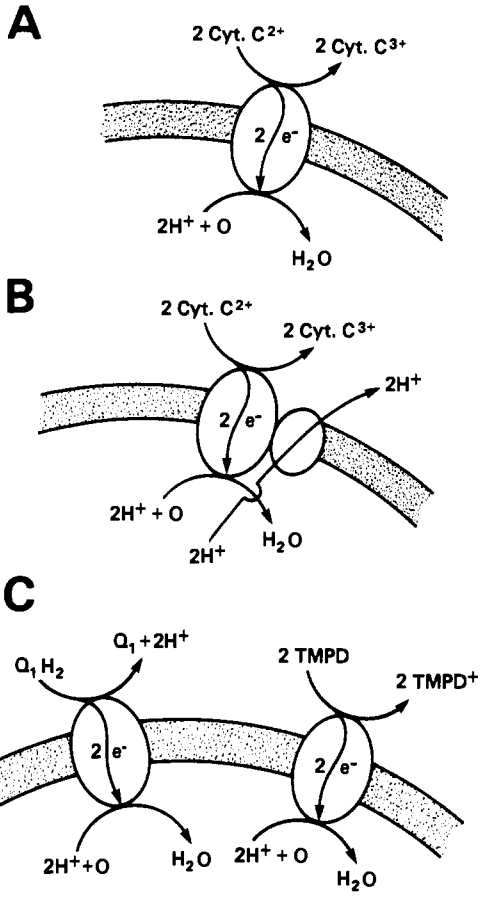

FIGURE 13: Models for $\Delta\psi$ and ΔpH generation by various terminal oxidases. (A) Mitochondrial aa_3-type oxidase, as discussed by Mitchell (1968). $\Delta\psi$ (interior negative) is generated as a result of vectorial electron flow from cytochrome *c* on the outer surface of the membrane to the inner surface. ΔpH (interior alkaline) is established because protons are consumed on the inner surface of the membrane during reduction of molecular oxygen to form water. (B) Mitochondrial (Wikström, 1981), *P. denitrificans* (Solioz et al., 1982), PS-3 (Sone & Hinkle, 1982), and *T. thermophilus* (Sone et al., 1983) terminal oxidases as vectorial proton pumps. In addition to vectorial electron flow and scalar consumption of protons on the inner surface of the membrane to form water, these oxidases are postulated to translocate protons vectorially, as indicated. (C) Cytochrome *o* oxidase from *E. coli* with Q_1H_2 or TMPD as substrates. In both cases, vectorial electron flow from the outer to the inner surface of the membrane generates $\Delta\psi$ (interior negative). During Q_1H_2 oxidation, ΔpH (interior alkaline) is generated due to scalar reactions leading to the appearance of protons in the external medium and consumption of protons on the inner surface of the membrane. During TMPD oxidation, on the other hand, proton consumption occurs on the inner surface of the membrane, but protons are not generated on the outer surface. See text for additional discussion.

between proton appearance in the external medium and oxygen consumption should exceed 2, the theoretical value expected for the scalar release of protons from Q_1H_2 on the external surface. As demonstrated, this is not the case. Rather, H^+:O ratios of less than 2 are consistently observed during Q_1H_2 oxidation. Moreover, with right-side-out vesicles from *E. coli* GR19N which contain cytochrome *o* exclusively as a terminal oxidase, both D-lactate and Q_1H_2 yield H^+:O ratios approximating 2 (unpublished results). In addition, electron flow from D-lactate dehydrogenase on the inner surface of the membrane (Short et al., 1975; Owen & Kaback, 1978, 1979a,b) to ferricyanide in the external medium occurs with a H^+:e^- ratio of 1 in the presence of cyanide, and no $\Delta\psi$ is generated.

Another approach to the problem is utilization of electron donors that do not carry protons. By this means, scalar release

of protons on the external surface is avoided, and extrusion of protons by the oxidase should be readily demonstrated. Unfortunately, *E. coli* cytochrome *o* does not utilize cytochrome *c*, an ideal electron donor for this purpose (Wikström, 1981; Solioz et al., 1982; Sone & Hinkle, 1982). However, TMPD is a substrate of the oxidase, and at neutral pH, it carries only about 0.3 proton/mol (Papa et al., 1983b). As shown (cf. Figure 10), oxidation of TMPD leads to slight alkalinization of the external medium, rather than acidification, an effect that is markedly accentuated by addition of the protonphore CCCP. The most straightforward explanation for the observations is that oxidation of TMPD by cytochrome *o* leads to vectorial electron flow through the oxidase with generation of $\Delta\psi$ (interior negative) and consumption of protons from the internal medium with generation of ΔpH (interior alkaline). Since few scalar protons are released from TMPD, little change in external pH is observed until CCCP is added, at which point the medium becomes alkaline because protons are driven into the proteoliposomes in response to the $\Delta\bar{\mu}_{H^+}$ that has been established (Figure 13C). In conclusion, therefore, although it is impossible to provide definitive evidence that the *o*-type cytochrome oxidase from *E. coli* does not catalyze proton translocation, the evidence presently available is consistent with the original concept of Mitchell (1968), as applied to the *E. coli* respiratory chain by Poole & Haddock (1975).

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Registry No. Q_1H_2, 52590-98-4; TMPD, 100-22-1; DAD, 3102-87-2; PMS, 299-11-6; DCI, 956-48-9; HQNO, 341-88-8; UHDBT, 43152-58-5; KCN, 151-50-8; cytochrome *b*-558, 9064-78-2; cytochrome *b*-563, 37265-01-3; oxidase, 9035-73-8; hydrogen ion, 12408-02-5.

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Modulation of Sarcoplasmic Reticulum Ca^{2+}-Pump Activity by Membrane Fluidity†

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ABSTRACT: Intramolecular excimerization of 1,3-di-1-pyrenylpropane [Py(3)Py] was used to assess the fluidity of sarcoplasmic reticulum membranes (SR); on the basis of the spectral data, the probe incorporates completely inside the membrane probably somewhere close to the polar head groups of phospholipid molecules, however not in the very hydrophobic core. The excimerization rate is very sensitive to lipid phase transitions, as revealed by thermal profiles of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) bilayers. Cholesterol abolishes pretransitions and broadens the thermal profiles of the main transitions which vanish completely at 50 mol % sterol. Excimer formation in liposomes of SR total lipid extracts does not show any sharp transitions, as in the case of DMPC and DPPC. However, the plots display discontinuities at about 20 °C which are broadened by cholesterol and not observed at 50 mol % sterol. Also cholesterol has been incorporated in native SR membranes by an exchange technique allowing progressive enrichment without changing the phospholipid/protein molar ratio. As in liposomes, discontinuities of excimer formation at 20 °C are broadened by cholesterol enrichment. The full activity of uncoupled Ca^{2+}-ATPase is only affected by cholesterol above a molar ratio to phospholipid of 0.4. However, a significant decrease in activity (about 20%) is only noticed at a ratio of 0.6 (the highest technically achieved); at this ratio, about 28 lipid molecules per Ca^{2+}-ATPase are expected to be relatively free from cholesterol interaction. The vesicle structure is still intact at this high ratio, as judged from the absence of basal activity (not Ca^{2+} stimulated). However, the sterol significantly decreases to about 60% the energetic efficiency of Ca^{2+} pumping (Ca^{2+}/ATP ratio).

Sarcoplasmic reticulum membranes (SR)[1] have been extensively characterized in their structural and functional aspects (Weber et al., 1973; Hasselbach, 1979; Tada et al., 1978). The major protein, the Ca^{++}-pump enzyme, is intrinsically associated with membrane lipids which greatly influence the enzyme activity (Martonosi et al., 1971; Bennett et al., 1980; Johannsson et al., 1981a). Lipids in contact with the ATPase enzyme modulate its function through physical interactions (Bennett et al., 1980; Johannsson et al., 1981a), including changing membrane fluidity. Thus, Ca^{2+} translocation across SR membrane and molecular mechanisms associated to energy transductions between the electroosmotic energy of Ca^{2+} gradients and chemical energy of ATP may be modulated by lipid–protein interactions, presumably affected by membrane fluidity. Cholesterol has a condensing effect on the acyl chains of bilayers in the fluid state (Houslay & Stanley, 1982). When forced to interact with some membrane proteins, it has a strong inhibitory effect on their function (Warren et al., 1975). Cholesterol is normally excluded from direct contact with the Ca^{2+}-ATPase enzyme (Bennett et al., 1975; Johannsson et al., 1981a; Simmonds et al., 1982) and excluded from other membrane enzymes as well (Houslay & Stanley, 1982). Thus, cholesterol in moderate concentrations does not affect significantly the overall Ca^{2+}-ATPase activity. This work deals with an attempt to characterize the effect of cholesterol on membrane fluidity and the energetic coupling of Ca^{2+} pumping.

Materials and Methods

Fragmented sarcoplasmic reticulum from rabbit white muscles was prepared according to Madeira & Antunes-Madeira (1976), although isolation and ressuspension media always contained 2.5 mM DTT and 10 μM PMS. Protein was normally determined by the biuret method (Gornall et al., 1951). Some preparations were assayed by the Lowry procedure (Lowry et al., 1951) calibrated against the biuret analysis.

Lipids of SR were extracted by the method of Madeira & Antunes-Madeira (1976). Phospholipids were quantitated by measuring the inorganic phosphate (Bartlett, 1959), after hydrolysis of extracts at 180 °C in 70% $HClO_4$ (Böttcher et al., 1961). Cholesterol in lipid extracts was assayed by the

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[1] Abbreviations: ATP, adenosine triphosphate; Ca^{2+}-ATPase, (Ca^{2+} + Mg^{2+})-dependent ATP phosphohydrolase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; PMS, phenylmethanesulfonyl fluoride; Py, pyrene; $PyCH_2OCH_3$, (1-pyrenylmethyl) methyl ether; Py(3)Py, 1,3-di-1-pyrenylpropane; SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)-aminomethane.

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